Drosophila metamorphosis: **The only way is USP?**

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The steroid insect molting hormone 20-hydroxyecdysone is believed to control critical aspects of development and reproduction through a heterodimeric receptor comprising the Ecdysone Receptor and the Ultraspiracle proteins. Recent findings suggest that other hormones and receptors might also be involved.

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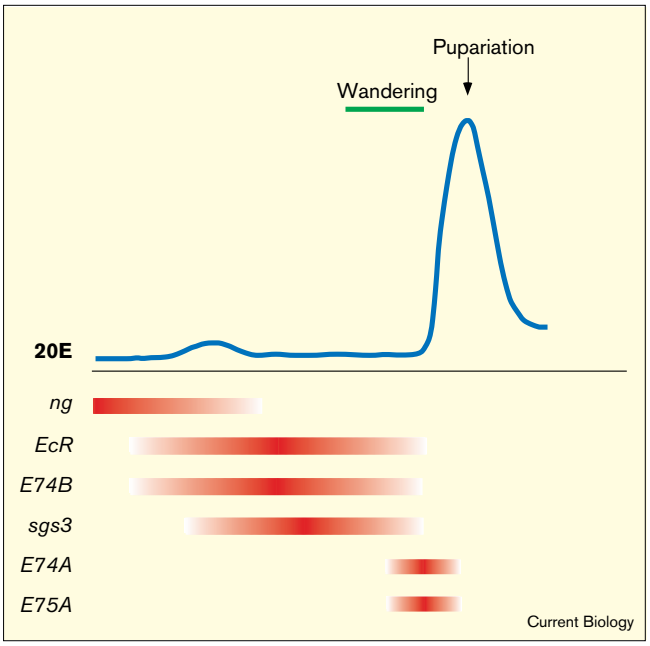
Larval molting, metamorphosis and reproduction in insects and other arthropods are controlled by a class of steroid hormones known as ecdysteroids. The active ecdysteroid in most insects appears to be 20-hydroxyecdysone. Like other steroid hormones and small hydrophobic hormones, ecdysteroids act by binding to receptors of the nuclear hormone receptor superfamily, which are ligand-dependent transcription factors. In *Drosophila*, genetic regulatory hierarchies underlying the ecdysteroid response have been well characterized [1]. Binding of 20-hydroxyecdysone to the ecdysteroid receptor activates the expression of a small group of early genes including *Broad-Complex* (*BR-C*), *E74* and *E75*. These genes encode transcription factors that attenuate their own activity while driving the expression of a large group of late genes [1]. Aspects of this response hierarchy have been shown to be conserved in a number of other species, including the tobacco hornworm, *Manduca sexta*, and the yellow fever mosquito, *Aedes aegypti* [2,3].

Studies on the ecdysteroid receptor indicate that this receptor is a heterodimer between two nuclear hormone receptors, Ecdysone Receptor (EcR) [4] and Ultraspiracle (USP) [5], the *Drosophila* homolog of the mammalian nuclear hormone receptor heterodimeric partner RXR. Binding to the ecdysteroid response elements is dependent upon the presence of both EcR and USP, and is, under appropriate conditions, hormone dependent [6,7]. Activation of transcription from the ecdysteroid response elements in heterologous cells similarly requires both EcR and USP [6,7]. Localization of EcR and USP proteins on polytene chromosomes suggests that these proteins are colocalized to the sites of ecdysteroid-responsive chromosome puffs, which represent sites of transcriptional activity [6,7]. Although these findings indicate that the active ecdysteroid receptor in *Drosophila* is likely to be a heterodimer of the EcR and USP proteins, the presence of numerous orphan receptors — so-called because their putative ligands are as yet unknown — raises the important question of whether EcR and USP might also be able to form heterodimers with other receptor-like proteins. Indeed, the finding that *usp* does not appear to be required in a cell-autonomous fashion for metamorphosis [8], and the intriguing differences between *EcR* and *usp* embryonic phenotypes, clearly suggest that the functions of *EcR* and *usp* may not be inextricably tied to one another [8,9]. These questions have come to the fore as recent findings on *usp*, the orphan receptor DHR78 and the action of hormones during the mid-third instar have prompted a more thorough examination of the hormones and receptors responsible for controlling late-larval development (Figure 1).

The most recent findings address the function of *usp* in a more systematic fashion than previous studies [10]. Animals mutant for *usp* that were rescued from early lethality by a heat-shock-inducible transgene survive to the third instar but do not undergo metamorphosis. These mutants fail to leave the food and wander in preparation for metamorphosis and also fail to express the early response genes *E74A*, *E75A* and *BR-C* in response to the characteristic late-larval, high-titer ecdysteroid pulse. Unexpectedly, however, the mid-third instar ecdysteroid response hierarchy, which sets the stage for the late-larval response, appears to be unaffected by the loss of *usp*. *EcR*, *E74B* and *BR-C* are expressed normally and the induction of the *salivary gland secretion* (*sgs*) genes, which encode the salivary glue proteins, occurs at the same time in the mid-third instar as in control animals. These findings suggest that USP may not be a component of the receptor controlling the mid-third instar ecdysteroid response.

There are, however, at least two alternative explanations for these above findings that must be considered. First is the possibility that a low, undetectable level of USP remains from the earlier heat shock and is sufficient to mediate the mid-third instar response. Indeed, our own findings on perdurance of EcR protein following the generation of mitotic clones suggest that significant persistence of low levels of receptor could be occurring (M.B. and W.A.S., unpublished observations). A second explanation, while less simple, also cannot be ruled out. Like the thyroid hormone receptor, the ecdysteroid receptor appears to act as a hormone-independent repressor of target gene expression [4,11,12]. In the light of this observation, it is clear that loss of the functional receptor may not be equivalent to loss of hormonal activation in all cases. Instead, if the repressive effect of the unliganded

Figure 1



Ecdysteroid-responsive gene expression during late larval development. The composite relative ecdysteroid titer (in 20-hydroxyecdysone (20E) equivalents, adapted from [17]), critical developmental events and the expression of representative ecdysteroid-responsive genes are shown.

receptor is required in order to keep some ecdysteroid-responsive genes transcriptionally inactive, receptor inactivation may instead replicate aspects of what would normally be a hormone-dependent de-repression (Figure 2). Indeed, this hypothesis would also explain a previously mysterious finding with regard to the role of ecdysteroids and receptors in *Drosophila* eye differentiation during the third instar: although progression of the morphogenetic furrow — a wave of differentiation that sweeps across the eye — is dependent upon ecdysteroids, loss of *usp* in mitotic clones actually appears to accelerate this progression [13,14]. Despite these caveats, it is clearly worth investigating the possibility that the ecdysteroid response during the mid-third instar may naturally proceed in the absence of USP, and that there may be a USP-independent ecdysteroid receptor.

What might that receptor be? Although certain *EcR* mutants survive to the third instar and exhibit defects in wandering behavior and pupariation [9], residual EcR activity from the hypomorphic alleles in these mutants prevents a clear interpretation of whether EcR is required for the mid-third instar response. Thus, an alternative receptor could be a heterodimer of EcR and some other protein, or an EcR homodimer or, instead, a completely new type of ecdysteroid receptor. While the identity of such a receptor, should it exist, remains to be seen, there is no paucity of candidates. The prevalence of orphan receptors within the ecdysteroid response pathways has led to the proposal of a number of models for their possible function. Several studies suggest that the orphans may modulate the ecdysteroid response by competition with the ecdysteroid receptor at the level of DNA binding or by competition for heterodimerization with EcR or USP. It seems unlikely, however, that these findings reflect the full spectrum of orphan receptor function and the possibility that one or more of the orphans might be regulated directly by ligand binding remains attractive [15].

Recent findings on the orphan receptor DHR78, in particular, suggest that this protein may play a role in the mid-third instar transition, perhaps as an ecdysteroid receptor. DHR78 has been shown to bind to ecdysteroid response elements and to loci on polytene chromosomes corresponding to sites bound by EcR and USP [16]. *DHR78* mutants initiate wandering but fail to express *EcR*, *E74B*, *BR-C* and *sgs* genes during the mid-third instar [16]. The possibility that loss of *DHR78* indirectly affects the mid-third instar transition by affecting the earlier molt cannot be ruled out, but the simplest interpretation is that *DHR78* is required at this important and poorly understood transition. The observation that *DHR78* over-expression does not have substantial phenotypic effects is also consistent with the possibility that its action is dependent on a ligand [16]. As pointed out by the authors, mechanisms such as covalent modification and cofactor interaction, which limit the activity of other, ligand-independent transcription factors, could of course be invoked. Given that *usp* is not required at this transition, however, these findings are sufficiently intriguing that one may be certain that the potential ligand modulation of DHR78 will be thoroughly investigated in future studies.

What might the ligand for the mid-third instar transition be? It has generally been accepted that the ligand is an ecdysteroid, largely based on the detection of a modest mid-third instar ecdysteroid pulse in apparent synchrony with mid-third instar changes in gene expression [17,18]. Moreover, expression of the *diptericin* gene, which normally occurs at the same time during the mid-third instar as the switch in salivary gland gene expression from *nested gene* (*ng*) expression to salivary glue protein expression, is dependent upon ecdysteroids [19]. At this time, ecdysone levels are high while 20-hydroxyecdysone levels remain relatively low. This situation changes at the time of pupariation when its significantly more active derivative 20-hydroxyecdysone becomes the predominant hormone. Although ecdysone has long been considered primarily as a precursor of 20-hydroxyecdysone, it does have biological activity, and recent studies have provided the best evidence to date that it is a physiologically significant hormone. Investigating the development of the *Manduca* eye, Champlin and Truman [20] have shown that ecdysone is effective in promoting the

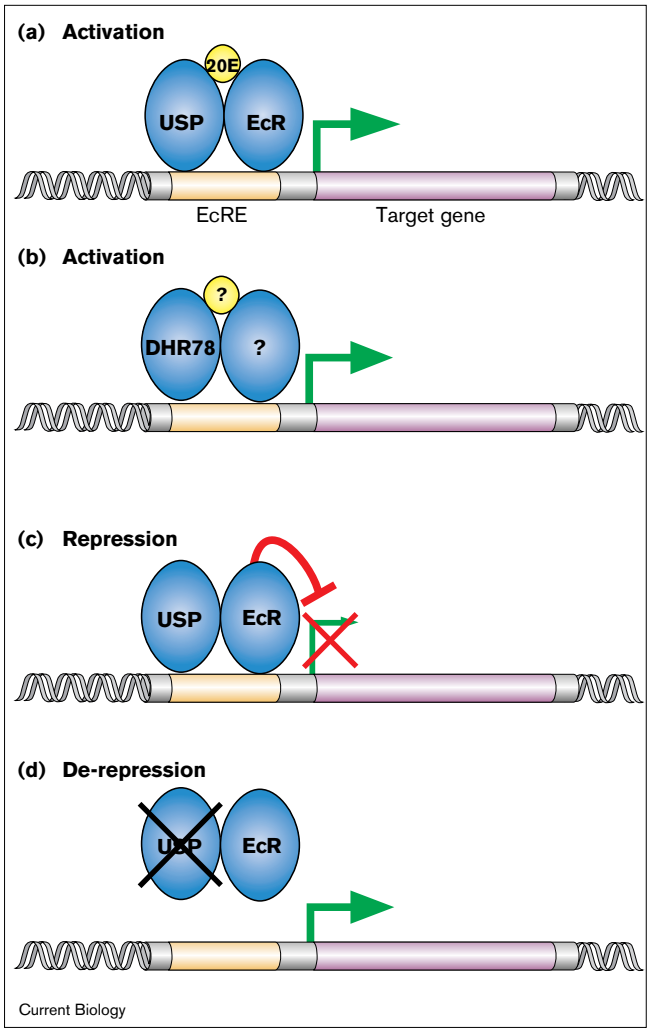
progression of morphogenesis in the eye anlagen during the final instar, and that 20-hydroxyecdysone is capable of promoting a distinct terminal differentiative response. These results suggest the possibility that ecdysone may play a significant role in at least some events associated with the mid-third instar transition in both *Drosophila* and *Manduca*.

While it will clearly be important to determine the extent to which the effects of ecdysone may be distinguishable from those of 20-hydroxyecdysone, the broader significance of this question depends critically on whether there is in fact a second ecdysteroid receptor with distinct or partially overlapping ligand specificity. On the basis of competitive binding studies and the correlation between binding coefficients and biologically active hormone titers, it has long been accepted that a single receptor could mediate the effects of both 20-hydroxyecdysone and ecdysone [21,22]. Indeed, these most recent results do not directly contradict that hypothesis: either 20-hydroxyecdysone, at low concentration, or ecdysone is capable of carrying out the proliferative response in the *Manduca* eye [20]. It is intriguing, however, that the relative efficacy of the two hormones is considerably more similar in this assay than in previous assays and considerably more similar than might be expected on the basis of binding coefficients, and it is also interesting that 20-hydroxyecdysone is able to mediate a distinct effect. Together, these findings prompt a careful re-examination of the existing presumptions concerning the identity of biologically active ecdysteroids and their receptors.

At the same time that we are led to ask whether there may be an ecdysteroid response in the absence of *usp* expression, evidence increasingly suggests that USP may have functions extending beyond its partnership with EcR in the formation of an ecdysteroid receptor. One of the most elusive goals of insect endocrinology has been unraveling the mysteries of juvenile hormone (JH), the small hydrophobic molecule responsible for modulating ecdy-steroid response as well as carrying out other functions during development and reproduction. On the basis of its structural resemblance to other nuclear hormone receptor ligands, and to retinoic acid in particular, it has long seemed likely that JH might act through a nuclear hormone receptor, but a receptor for JH has proven maddeningly elusive. Recently, new evidence indicating that JH can bind to USP and stimulate multimerization of USP *in vitro* and in yeast cells has suggested the possibility that USP might be a critical component of a JH receptor [23].

Such a hypothesis finds support in the analysis of *usp* mutants. One of the best-characterized actions of JH is its ability to modulate ecdysteroid-dependent cuticle production, with larval cuticle forming in the presence of JH and pupal cuticle in its absence [24]. The formation of a supernumerary larval cuticle at the time of pupariation in *usp* mutants suggests that *usp* mutations disrupt this modulation of ecdysteroid response by JH [10]. The possibility that USP can act as a JH receptor clearly presents the attractive possibility that JH might directly modulate the activity of the familiar heterodimeric EcR–USP ecdysteroid receptor complex. Nonetheless, it is too soon to speculate whether JH might act via the EcR–USP heterodimer, a USP homodimer, or a heterodimer containing USP and some other nuclear hormone receptor, or

Figure 2



Regulation of gene expression by ecdysteroid receptors. **(a)** Binding of 20-hydroxyecdysone (20E) to an EcR–USP heterodimer allows this complex to bind to ecdysteroid response elements (EcREs) and activate target gene expression. **(b)** DHR78 has been shown to bind to similar elements and its ability to mediate gene expression during the mid-third instar may require binding to either an unidentified ligand or another receptor. **(c)** EcR–USP heterodimers not bound to 20-hydroxyecdysone may repress transcription in a manner similar to the thyroid hormone receptor. **(d)** Loss of one or both components of the ecdysteroid receptor could therefore lead to activation of target gene expression by abolishing the repressive activity of the unbound receptor.

whether putative USP-mediated effects of JH would occur through ecdysteroid response elements or some other response element.

What once seemed a relatively simple path from a hormonal signal to an initial target gene response has been revealed by these recent findings to be far more complex than anticipated. Whatever the results of the upcoming experiments, these latest findings on the ecdysteroid response and the ecdysteroid receptor make it clear that there are still many mysteries to be solved.

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